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Occurrence and Resistance to Antibiotics of *Campylobacter* spp. in Retail Raw Sheep and Goat Meat in Shahr-e Kord, Iran

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Abstract: This study was conducted to determine the prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from retail raw sheep and goat meats in Iran. From July 2009 to March 2010, a total of 224 raw meat samples from sheep (n = 114) and goat (n = 110) were purchased from randomly selected retail outlets in Shahr-e Kord, Iran and were evaluated for the presence of *Campylobacter*. Using cultural method, 22 of 224 meat samples (9.8%) were contaminated with *Campylobacter*. The prevalence of *Campylobacter* spp. in sheep and goat meat samples were 13.2% and 6.4%, respectively. The most prevalence *Campylobacter* species isolated from meat samples was *Campylobacter jejuni* (81.8%); the remaining isolates were *Campylobacter coli* (18.2%). The PCR assay could identify 4 *Campylobacter*-contaminated samples that were negative using the cultural method. Susceptibilities of 22 *Campylobacter* isolates were determined for ten antimicrobial drugs using the disk diffusion assay. Resistance to tetracycline was the most common finding (63.6%), followed by resistance to ciprofloxacin (45.5%), enrofloxacin (31.8%), nalidixic acid (31.8%) and ampicillin (13.6%). All of the isolates were susceptible to amoxicillin, erythromycin, gentamicin and chloramphenicol. More epidemiological studies are needed in order to determine the possible role of sheep and goat as a source and/or reservoir of *Campylobacter* spp.

Key words: *Campylobacter* · Raw meat · Sheep · Goat · Antimicrobial resistance

INTRODUCTION

Campylobacters are relatively 'new' zoonotic pathogens as routine culture from clinical specimens only became possible in the late 1970s [1]. *Campylobacter* is the leading cause of zoonotic enteric infections in many countries and the public health burden of campylobacteriosis is increasing [2]. *Campylobacter* are Gram negative, slender, spiral curved rods having dimensions of 0.2 m to 0.8 m wide and 0.5 m to 5 m long. Extremely rapid, darting, reciprocating motility can be seen with a phase contrast microscope, with comma-shaped, S, or gull wing-shaped cells and are microaerophilic, growing best at gaseous dioxide and 85% nitrogen [3]. Of the 17 species within the genus *Campylobacter* [4], *Campylobacter jejuni* and *Campylobacter coli*, have been recognized as the primary causative agents of bacterial human foodborne gastroenteritis in both industrialized and developing countries [5].

A number of transmission vehicles and risk factors have been implicated in previously reported case control studies that examined predisposing data from human *Campylobacter* cases and outbreaks. Food and particularly poultry, is involved in about 80% of cases of human campylobacteriosis [3,6-7]. *Campylobacter* species readily colonize the gastrointestinal tracts of domestic, fecal and wild animals and while they rarely cause clinical disease in food animals, they can produce severe acute gastroenteritis in humans [4]. Several studies have shown *Campylobacter* contamination of faecal samples from beef, pork and poultry, of meat from pork, beef, turkey, shellfish and of sheep's liver [8-10]. Consumption of inadequately treated water, raw milk and various meats, in addition to contact with pets and farm animals, has been implicated in outbreaks of campylobacteriosis [4].

Treatment with antibiotics for uncomplicated *Campylobacter* infection is rarely indicated. However, antimicrobial resistance to clinically important drugs used

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for treatment (especially macrolides and fluoroquinolones) is increasingly reported for campylobacters. There is evidence that patients infected with antibiotic-resistant strains suffer worse outcomes (invasive illness or death) than those infected with sensitive strains [11]. Antimicrobial resistance has emerged among *Campylobacter* mainly as a consequence of the use of antimicrobial agents, especially fluoroquinolones, macrolides and tetracyclines in food animal production [12-16]. This underlines the need to limit the use of antimicrobials in veterinary and medical clinical practice to limit the occurrence of resistance [1].

Currently, there is limited information regarding the prevalence and antimicrobial susceptibility patterns of *Campylobacter* in raw meat in Iran. The present study was conducted to determine the prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from retail raw sheep and goat meat in Shahr-e Kord, Iran. The antimicrobial agents tested in this study are widely used to treat infections in people and in food animals in Iran.

MATERIALS AND METHODS

Sample Collection: From July 2009 to March 2010, a total of 224 raw meat samples from sheep (n = 114) and goat (n = 110) were purchased unpacked from randomly selected butcheries in Shahr-e Kord, Iran. All samples were placed in separate sterile plastic bags to prevent spilling and cross contamination and were immediately transported to the laboratory in a cooler with ice packs.

Microbiological Analysis: The samples were processed immediately upon arrival using aseptic techniques. Of each meat sample, 25 g was homogenized and transferred to 225 mL of Preston enrichment broth base containing *Campylobacter* selective supplement IV (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood. After inoculation at 42°C for 24 h in a microaerophilic condition (85% N_2, 10% CO_2, 5% O_2), 0.1 mL of the enrichment was then streaked onto Campylobacter selective agar base (HiMedia Laboratories, Mumbai, India) supplemented with an antibiotic supplement for the selective isolation of *Campylobacter* species (HiMedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood and incubated at 42°C for 48 h under the same condition. One presumptive *Campylobacter* colony from each selective agar plate was subcultured and identification of presumptive *Campylobacter* species was performed using standard microbiological and biochemical procedures including Gram staining, production of catalase, oxidase, hippurate hydrolysis, urease activity, indoxyl acetate hydrolysis and susceptibility to cephalotin [10-17].

DNA Extraction and PCR Conditions: DNA from 224 samples was extracted from Preston broth after the enrichment step using a Genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol. The PCR procedures used in this study have been described previously [18]. Three genes selected for the identification of the *Campylobacter* spp. *C. jejuni* and *C. coli* were the *16S rRNA* gene [19], the *mapA* gene [20] and the *ceuE* gene [21], respectively. The sequences of the three sets of primers used for gene amplification are presented in Table 1. Amplification reactions were performed in a 30 μL mixture containing 0.6 U Taq polymerase (Fermentas, GmbH, Germany), 100 μmol l^{-1} of each dNTP, 0.11 μmol l^{-1} of MD16S1 and MD16S2 primers and 0.42 μmol l^{-1} of MdmapA1, MdmapA2, COL3 and MDCOL2 primers in the Fermentas buffer (Fermentas, GmbH, Germany). Amplification reactions were carried out using a DNA thermal cycler (Master Cycle Gradiant, Eppendrof, Germany) with the following program: one cycle of 10 min at 95°C, 35 cycles each consisting of 30 s at 95°C, 1 min and 30 s at 59°C, 1 min at 72°C and a final extension step of 10 min at 72°C. The amplification generated 857 bp, 589 bp and 462 bp DNA fragments corresponding to the *Campylobacter* genus, *C. jejuni* and *C. coli*, respectively. *C. coli* (ATCC 33559) and *C. jejuni* (ATCC 33560) were used as the positive controls and DNase free water was used as the negative control. The PCR products were stained with 1% solution of ethidium bromide and visualized under UV light after gel electrophoresis on 1.5% agarose.

Table 1: Primers for polymerase chain reaction (PCR) amplification of campylobacterial DNA for identification DNA

Organism	Primer	PCR product (bp)	Sequence
Campylobacter spp.	*16SrRNA*	857	5' ATC TAA TGG CTT AAC CAT TAA AC 3'
			5' GGA CGG TAA CTA GTT TAG TAT T 3'
Campylobacter jejuni	*mapA*	589	5' CTA TTT TAT TTT TGA GTG CTT GTG 3'
			5' GCT TTA TTT GCC ATT TGT TTT ATT A 3'
Campylobacter coli	*ceuE*	462	5' AAT TGA AAA TTG CTC CAA CTA TG 3'
			5' TGA TTT TAT TAT TTG TAG CAG CG 3'

Antimicrobial Susceptibility Testing: One strain from each *Campylobacter*-positive sample was selected for susceptibility tests. Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) supplemented with 5% defibrinated sheep blood, according to the Clinical Laboratory Standards Institute [22]. The following antimicrobial impregnated disks (HiMedia Laboratories, Mumbai, India) were used: nalidixic acid (30 μg), ciprofloxacin (15 μg), erythromycin (15 μg), tetracycline (15 μg), streptomycin (30 μg), gentamicin (10 μg), amoxicillin (30 μg), ampicillin (10 μg), chloramphenicol (30 μg) and enrofloxacin (10 μg). After incubation at 42°C for 48 h in a microaerophilic atmosphere, the susceptibility of the *Campylobacter* spp. to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI (22). *Staphylococcus aureus* and *Escherichia coli* were used as quality control organisms in antimicrobial susceptibility determination.

Statistical Analysis: Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp. Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc. Chicago, IL, USA), chi-square test and fisher's exact two-tailed test analysis were performed and differences were considered significant at values of $P < 0.05$.

RESULTS

Table 2 shows the prevalence of *Campylobacter* spp. isolated from camel, beef, lamb and goat meat in Shahr-e Kord, Iran. Using cultural techniques, 22 of 224 meat samples (9.8%) were found to be contaminated with *Campylobacter*. The prevalence of *Campylobacter* spp. in sheep meat and goat meat were found 13.2 and 6.4%, respectively. No significant differences were found between sheep meat and goat meat. The most prevalence rate of *Campylobacter* species isolated from meat samples was *C. jejuni* (81.8%); the remaining isolates were *C. coli* (18.2%).

Overall, 22 raw sheep and goat meat samples were positive for *Campylobacter* spp. using both the cultural method and the PCR assay (Table 2). The PCR assay could identify 4 *Campylobacter*-contaminated raw meat samples that were negative using the cultural method.

The resistance pattern of *Campylobacter* isolates to 10 antimicrobial agents tested in this study is shown in Table 3. Overall, 21 of 22 *Campylobacter* isolates (95.5%) were resistant to one or more antimicrobial agent. Five strains (22.7%) were resistant to single antibiotic and 11 strains (50.0%) showed resistance to two antimicrobial agents. Multiresistance which was defined as resistance to three or more of drug tested was found in 22.7% of *Campylobacter* strains. Resistance to tetracycline and ciprofloxacin was the most common finding (63.6%),

Table 2: Prevalence of *Campylobacter* spp. isolated from sheep and goat meat in Shahr-e Kord, Iran, using cultural technique and polymerase chain reaction (PCR) assay

Sources of samples	No. of samples	Number (%) of samples positive using cultural technique			Number (%) of samples positive using PCR assay			
		Campylobacter spp.	*C. jejuni*	*C. coli*	No. of samples	*Campylobacter* spp.	*C. jejuni*	*C. coli*
Sheep	114	15 (13.2)	11 (73.3)	4 (26.7)	114	18 (15.8)	14 (77.8)	4 (22.2)
Goat	110	7 (6.4)	7 (100)	0 (0.0)	110	8 (7.3)	7 (87.5)	1 (12.5)
Total	224	22 (9.8)	18 (81.8)	4 (18.2)	224	26 (11.6)	21 (80.8)	5 (19.2)

*Results expressed as the number of *Campylobacter*-positive samples / number of samples analyzed (%).

Table 3: Antimicrobial resistance profiles of *Campylobacter* strains isolated from sheep and goat meat in Shahr-e Kord, Iran

Antimicrobial agent	*Campylobacter* spp.(N = 22)	*Campylobacter jejuni* (N = 18)	*Campylobacter coli* (N = 4)
Amoxicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ampicillin	3 (13.6%)	2 (11.1%)	1 (25.0%)
Chloramphenicol	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ciprofloxacin	10 (45.5%)	8 (44.4%)	2 (50.0%)
Enrofloxacin	7 (31.8%)	7 (100%)	0 (0.0%)
Erythromycin	0 (0.0%)	0 (0.0%)	0 (0.0%)
Gentamicin	0 (0.0%)	0 (0.0%)	0 (0.0%)
Nalidixic acid	7 (31.8%)	6 (33.3%)	1 (25.0%)
Streptomycin	0 (0.0%)	0 (0.0%)	0 (0.0%)
Tetracycline	14 (63.6%)	13 (72.2%)	1 (25.0%)
Total			
Resistance to 1 antimicrobial	5 (22.7%)	4 (22.2%)	1 (25.0%)
Resistance to 2 antimicrobials	11 (50.0%)	9 (50.0%)	2 (50.0%)
Resistance to > 2 antimicrobials	5 (22.7%)	5 (27.8%)	0 (0.0%)

followed by resistance to ciprofloxacin (45.5%), enrofloxacin (31.8%), nalidexic acid (31.8%) and ampicillin (13.8%). All *Campylobacter* isolates were susceptible to amoxicillin, chloramphenicol, erythromycin, gentamicin and streptomycin. *Campylobacter coli* isolates were also susceptible to enrofloxacin.

DISCUSSION

In the present study, 13.2% and 6.4% of retail sheep and goat meat samples were *Campylobacter*-positive, respectively, which are comparable with those reported from Iran [23], Ireland (10), Ethiopia [24-25] and Switzerland [26], however, are higher than those reported from Australia [27] and Pakistan [28]. Higher prevalence of *Campylobacter*-positive samples in the present study may be due to cross-contamination during manual skinning, evisceration and processing in the slaughterhouse or insufficient hygiene during storage, transport and boning in the butcheries, particularly in small butcher shops where there may have been closer proximity to meat from other food animal species. *Campylobacter* present in the intestinal tract of animals represents a potential risk for the contamination of carcasses with *Campylobacter* depending on shedding patterns and hygienic manufacturing practices [29].

In this study, *C. jejuni* was the most prevalence *Campylobacter* species recovered from meat samples. *Campylobacter jejuni* has been reported to be the most frequent species recovered from food of animal origin specially poultry meat [8,24,28-29]. Our results on the prevalence of *C. jejuni* in raw meat are in agreement with data from other countries [10,26,28-30]. Variation in the prevalence of *Campylobacter* isolates from raw meat samples reported in other studies may be a result of different sampling techniques employed, seasonal effects and/or laboratory methodologies employed in different studies.

Isolation and identification of *Campylobacter* spp. have traditionally involved the use of selective culture media combined with biochemical tests. This method is expensive, laborious and time-consuming whereas PCR assay is fast and cost-effective [31]. In this study, *Campylobacter* was more detected by the PCR assay than the cultural method. This could be due to the higher analytical and diagnostic sensitivities of the PCR assay.

The results of antimicrobial susceptibility testing in the present study indicate that there is a high resistance of *Campylobacter* spp. to tetracycline, ciprofloxacin, nalidixic acid and enrofloxacin. These results are comparable to those reported by other investigators [9,15-16,23]. The results of antimicrobial resistance found in this study are correlated to antibiotics that are being used to treat infection in food animals in Iran. For example, tetracycline, ciprofloxacin and enrofloxacin which are closely related to ciprofloxacin are widely used for growth promotion and treatment of livestock [15-16]. Hence, the antimicrobial resistance profile of pathogenic food isolates can reflect the antimicrobial substances used for animal treatment. Tetracycline is the drug most often used in animal husbandry and is a drug of second choice in human medicine [16]. Due to the high number of antimicrobial-resistant isolates, we recommend that *in vitro* antimicrobial susceptibility testing of *Campylobacter* be performed and appropriate treatment be instituted especially for those cases of food borne campylobacteriosis with sever or prolonged symptoms or in immunocompromised patients.

In conclusion, the presence of *Campylobacter* in sheep and goat meat determined in the present study, suggests that sheep and goat may be a potential sources of *Campylobacter* spp. human infection due to this organisms. In order to protect public health, a series of control strategies should be developed and implemented at all stages of food production, system to eliminate contamination with this important human pathogen. More epidemiological studies are needed in order to determine the possible role of sheep and goat as a source and/or reservoir of *Campylobacter* spp.

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